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Shared effects of organic microcontaminants and environmental stressors on biofilms and invertebrates in impaired rivers☆

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ABSTRACT

Land use type, physical and chemical stressors, and organic microcontaminants were investigated for their effects on the biological communities (biofilms and invertebrates) in several Mediterranean rivers. The diversity of invertebrates, and the scores of the first principal component of a PCA performed with the diatom communities were the best descriptors of the distribution patterns of the biological communities against the river stressors. These two metrics decreased according to the progressive site impairment (associated to higher area of agricultural and urban-industrial, high water conductivity, higher dissolved organic carbon and dissolved inorganic nitrogen concentrations, and higher concentration of organic microcontaminants, particularly pharmaceutical and industrial compounds). The variance partition analyses (RDAs) attributed the major share (10%) of the biological communities' response to the environmental stressors (nutrients, altered discharge, dissolved organic matter), followed by the land use occupation (6%) and of the organic microcontaminants (2%). However, the variance shared by the three groups of descriptors was very high (41%), indicating that their simultaneous occurrence determined most of the variation in the biological communities.

1. Introduction

Rivers are net receivers of chemical stressors from anthropogenic origin, including organic matter and inorganic nutrients in excess (phosphorus, nitrogen), and many organic micropollutants (Meybeck, 2004) such as pesticides or industrial products. Rivers may receive other stressors that co-occur with these chemicals (Stevenson and Sabater, 2010). Amongst these stressors, habitat alteration, interruption of flow water regime, or higher water temperature, complicate the survival and life cycle of organisms, especially of those sensitive, and are at the base of local extinctions and the overall decrease of biodiversity (Dudgeon, 2010). These alterations perform as additional environmental filters (Poff, 1997; Angermeier and Winston, 1998; Malmqvist, 2002), and condition the composition and relative abundance of species in the riverine biological communities.

Persistent chemical pollution has been seen as a prevalent driver with respect to other stressors in impaired freshwater ecosystems (Malaj et al., 2014). Organic microcontaminants constitute complex mixtures that may differ according to the prevailing land uses, i.e., extensive agriculture, industrial activities, or human conurbations (Posthuma et al., 2008). The composition and concentration of micropollutants also vary between periods of the year depending on their use, and because of higher or lower water discharge (Petrovic et al., 2011). Their concentration may be enhanced or moderated according to the dilution capacity of the receiving river; arid and semiarid basins, but also those subjected to water abstraction (Barceló and Sabater, 2010), have low dilution capacity and are candidates to higher effects. Micropollutant effects

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not only depend on their concentration but also on the pollutants mixture (Altenburger et al., 2015) and their specific mode of action (Cleuvers, 2003). These organic microcontaminants may coincide with nutrients in excess, or with abundant dissolved organic matter, especially in systems heavily impacted by industrial or urban effluents (Hatt et al., 2004), making up a co-occurring number of stressors with complex effects on the biological communities (Segner et al., 2014).

The biota inhabiting freshwater ecosystems is the final receptor of this large diversity of influences. It was already shown long time ago that biological communities were modulated by chemical pressures, such as the dissolved organic matter (evaluated by means of the COD or TOC), as well as by nutrients in excess, or by heavy metal pollution (Margalef, 1960; Goodnight, 1956). These observations can be placed at the basis of the modern use of organisms and communities as indicators of the ecological status of ecosystems, with expressions such as water quality indices (Lecointe et al., 1993; Armitage et al., 1984) or multimetric approaches (e.g. Fore et al., 1996). Altogether, these applications are based on the evidence that bacteria, algae, invertebrates, or fish, had characteristic ways to respond to the occurring stressors. The specific responses of each group of organisms are related to their particular life cycle and habitat that they occupy, and translate in specific roles in the energy and matter flux in the ecosystem. Shorter life-cycle organisms (bacteria, algae) may respond to rapid changes occurring in the river environment, both physical (temperature, salinity, pH) and chemical (nutrient abundance, organic matter availability) and biological (grazing, predation). The ones occupying the interphase between water and sediments (biofilms) can be the most responsive to short-term changes afe¡ter these pressures (Blanck et al., 1988; Sabater et al., 2007). On the other hand, longer life-cycle organisms (invertebrates, fish) are able to integer the long-lasting changes produced in the environment in their physiological status and population dynamics, and may therefore be responsive to chemical alterations, but also to physical stressors (hydrological alterations, habitat impairment, altered temperature regime), and as such can be good indicators of persistent stress (Bonada et al., 2006; Boix et al., 2010; Johnson and Hering, 2009).

The recent awareness that organic anthropogenic substances may enter freshwaters in relatively high concentrations, and that they may affect biological communities (Beketov et al., 2013) has triggered huge efforts to understand their relevance for the ecosystem (Luo et al., 2014). At least some of these substances are able to bioaccumulate and propagate throughout the trophic web (Geyer et al., 2000; Arnot and Gobas, 2004), and may affect the composition and performance of biological communities (Muñoz et al., 2009; Ricart et al., 2010; Ginebreda et al., 2014). Their overall relevance when other disturbances also occur (organic matter or inorganic nutrients in excess, high concentrations of solutes such as chloride, hydrological pressures) is unclear, even despite recent indications of the potential relevance of microcontaminants (Liess et al., 2013). An obvious reason for these different perspectives is that matching potential effects to real consequences is not straightforward for the biological communities. Organisms are net receivers of influences at multiple spatial and temporal scales, and their ultimate response defines the carrying capacity of a system (Posthuma et al., 2014). Spatial influences range from basin-scale to reach-scale, that is, from general to local, and temporal scales may determine quick or accumulative changes, and translate differently to the organisms in relation to their size and life cycle. This complexity is obvious at the ecosystem level, where multiple vulnerabilities of biological communities co-exist according to their position in the trophic web and evolutionary traits (Segner et al., 2014).

Extensive field studies combining chemical and biological analyses allow for the definition of potential patterns and causes of distribution of the biological communities. Multivariate analyses allow performing joint ordination of several sets of physical, chemical and biological variables assembled from the field, and also to define the distribution patterns of organisms according to the driving pressures in a given set of sites. This is a correlational approach with recognised weaknesses (Legendre and Legendre, 1998), but also sufficiently powerful to define emerging patterns on the structure of ecological data (Legendre and Legendre, 1998). Such an approach may help understanding up to which degree the co-occurring stressors affect the community structure of microbial organisms (biofilms, including primary producers and heterotrophs), and invertebrate consumers (herbivores, detritivores and predators). While biofilms may be more sensitive to inorganic nutrients (Sabater et al., 2000) and to some organic micropollutants (e.g. herbicides, antibiotics; Proia et al., 2013; Pesce et al., 2011), invertebrates may better respond to habitat alteration as well as to other contaminants such as estrogenic substances, insecticides and even toxic nutrient concentrations (Muñoz et al., 2009; Camargo et al., 2005; De Castro-Català et al., 2013; Azevedo et al., 2015; Liess et al., 2013). No doubt, the joint response of ones and the others, if produced, may represent force of evidence of the impact of multiple disturbances on the river ecosystem, and hopefully might define situations and periods when these effects are more obvious. This principle has been recognized in several legislative frameworks (e.g. the European WFD, or the Clean Water Act in the US), where the response of these groups of organisms is considered complementary. The potential patterns derived from field-based exercises may shed light to potential causalities that in subsequent experimental approaches (Sabater et al., 2007; López-Doval et al., 2010) can be tested for their consistency and mechanisms.

In the present work, sites of four different Mediterranean basins were sampled for their physical (water flow, temperature, land uses), chemical (inorganic nutrients, conductivity, organic micropollutants), and biological (invertebrates, biofilm) descriptors. The patterns of biological communities with respect to the environmental and organic chemical pressures were explored in a variety of situations and multiplicity of scales. The main hypotheses were that, i) biofilms and invertebrates would show complementary responses to the co-occurring stressors, and ii) that the more pronounced effects on the biological communities would appear in sites with higher concentrations of organic micropollutants and higher degree of environmental impairment.

2. Material and methods

Four Mediterranean river basins of the Iberian Peninsula (The Llobregat, Ebro, Júcar and Guadalquivir) were used in this study. These basins drain a large part of the east and south Iberian Peninsula, and are mainly governed by Mediterranean climate (Sabater et al., 2009). A total of 19 sites were selected in the main course of the rivers: 5 in the Ebro (E1, E2, E3, E4 and E5), 5 in the Llobregat (L3, L4, L5, L6 and L7), 5 in the Júcar (J1, J2, J4, J6 and J7) and 4 in the Guadalquivir (G1, G2, G3 and G4) (see location in Fig. 1). Sites were selected in open areas of the middle course of the rivers. In all cases, water and biota samples were collected in riffle sections, where water was circulating. These sites did not receive direct inputs of WWTPs, tributary inputs, or spills. Yet, the sites were receiving a variety of diffuse and local inputs, depending on the land uses affecting the site, so their chemical and biological characteristics could be representative of the river segment. The sampling of water for chemical analyses and biological communities' characterization was performed at the end of the summer period in two consecutive years (2010 and 2011). The upstream sites in each of the basins were moderately affected, and the others downstream showed varying degree of impairment by agricultural, urban and industrial influences, hydrological alterations, and urbanization. The land uses corresponding to each subbasin area (the one draining to each site) was estimated by GIS analysis using the Corine Land Cover data base of 2006 (level 1 of classification of 4 classes, namely forested and seminatural, artificial (urban, industrial), agricultural, and others). The size of the cells was 100×100 m. Sub-basins were defined using the extension ArcHydro of ArcGIS. Inhabitants-equivalent to the presence of waste water treatment plants (WWTPs) in each subbasin were obtained from water authorities' websites.

2.1. Physical and Chemical measurements

Water flow in each sampling site was obtained from daily measurements from the nearest gauging station (data provided by local water agencies) during the 3-months period around the sampling date. In the case no direct measurements were available, the drainage-area ratio of each sub-basin was used to extrapolate the corresponding data. The variation coefficient of water flow (Q (cv)) was calculated, and used as an estimate of the flow variability in each site throughout the hydrological period. The physical and chemical variables measured in each site included dissolved oxygen (DO), water temperature (T), pH, and conductivity. Hand probes (WTW multiline 3310, and YSI ProODO, Yellow Springs, OH, USA) were used for the in situ measurements. Water samples for NH_4^+ and NO_3^- analyses (considered jointly as DIN), Dissolved Organic Carbon (DOC), and total phosphorus (TP), were filtered with glass fibre filters (Whatman GF/F) in situ and kept frozen at -20 °C until analysis. Nitrate was analysed by ion chromatography (DIONEXIC5000; Dionex Corporation, Sunnyvale, USA) and the concentrations of ammonium and phosphate were determined colorimetrically (Alliance-AMS Smartchem 140 spectrophotometer, Frepillon, France). The DOC concentration was analysed on a Shimadzu TOC-V CSH (Shimadzu Corporation, Kyoto, Japan).

At each site, grab water samples were taken for chemical analyses of the organic micropollutants. A total of 157 organic micropollutants were measured using previously published analytical methods based on gas chromatography-tandem mass spectrometry or liquid chromatography-tandem mass spectrometry for perfluorinated compounds (PFCs) (Onghena et al., 2012), pesticides (Masià et al., 2013), pharmaceuticals (Gros et al., 2009), endocrine disrupting chemicals (EDCs) and related compounds such as hormones, plasticisers, alkylphenolics, parabens, phosphate flame retardants, anticorrosion agents and bactericides (Gorga et al., 2013), and UV filters (Gago-Ferrero et al., 2013).

Once every compound was identified and quantified, the products were grouped for herbicides, organophosphate pesticides, fungicides, carbamates, neocotinoids, and pyretroids; antibiotics, analgesic and anti-inflammatories, anticoagulants, lipid regulators, histamines, b-blockers, antihypertensives, diuretics, antidiabetics, psychiatric drugs, and veterinary pharmaceuticals; alkylphenols, flame retardants, anticorrosives, bisphenol A (BPA); hormones, UV-filters, parabens, and bactericides. This classification was used for the exploration of potential relationships between the micro-contaminant classes and biota. Those showing significant correlation with the biological metrics were included in the multivariate analyses.

2.2. Biological components

We used data on community structure (composition and density) of the primary producers (diatoms) in the biofilm, as well as of the invertebrate community. These are commonly used as biological quality elements in monitoring schemes elsewhere. Biofilms and invertebrates were collected in the very same reaches, and simultaneously, to the places where the chemical samples and field measurements were also performed.

Biofilm collection, preparation for diatom examination, and counting followed described standard protocols (Kelly et al., 1998). The diatom community was used as the representative of the algal fraction of the biofilm; diatoms account for the majority of species within the whole algal species set in rivers (Round, 1981). To do so,

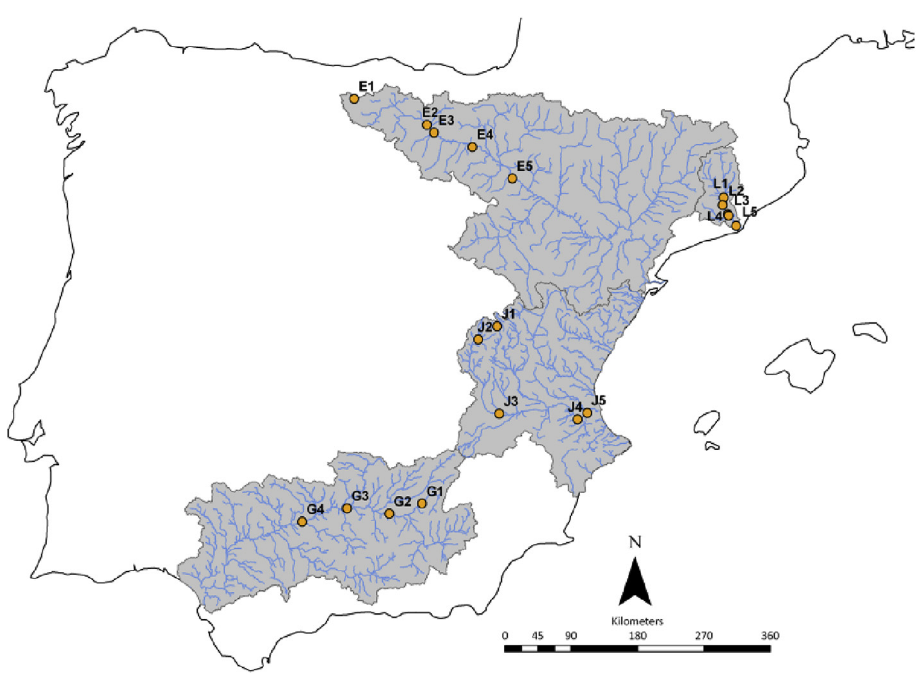

Fig. 1. The four Mediterranean basins studied (Iberian Peninsula), showing the location of the sites in each of the basins. Llobregat basin: L1, L2, L3, L4 and L5. Ebro basin: E1, E2, E3 E4 and E5. Júcar basin: J1, J2, J3, J4 and J5. Guadalquivir basin: G1, G2, G3 and G4.

up to 400 valves were determined at the species level on each slide by performing random transects under light microscopy (Nikon Eclipse 80i, Tokyo, Japan) using Nomarski differential interference contrast optics at a magnification of 1,000×. This is a standard procedure that allows considering all the present taxa in a sample together with their relative abundances (Kelly et al., 1998). The number of diatom taxa (S_D) in each sample, and the new variable first component (PC_{1D}) derived from the Principal component analysis (PCA) were used as descriptors of the diatom assemblages in the four basins. Data used in the PCA included both the species and their relative abundances in each sample, and the diatom taxa selected to participate in the analysis were those accounting for more than 1% of the relative abundance in at least three samples. In this case, the scores of the first component of the PCA (the first one having the most obvious biological meaning and the higher explained variance) were therefore used as the ordination of the whole diatom community structure according to the main environmental gradient in the whole set of cases.

Other biofilm measurements were also considered complementary to the diatom community composition. Biofilm material covering cobbles or stones were collected at each site (five replicates), and aliquots used for alkaline phosphatase activity (APA). Alkaline phosphatase activity (APA) is a measure of the ability of transformation of organic into inorganic phosphorus, mainly by bacteria and cyanobacteria. APA was determined using the substrate analogue 4-MUF- phosphatase (from Sigma Aldrich). Analytical details are given in Ricart et al. (2010) and Proia et al. (2013).

Invertebrate data were obtained from the analysis of sediment samples in each of the sites. Five samples were collected at random and invertebrates were sorted, counted, measured and identified under a dissecting microscope (Leica Stereomicroscope), in order to determine the community composition of the invertebrates (De Castro-Català et al., 2015). The identification was at the species level for nearly all groups of taxa (including Oligochaeta) with the exception of the Chironomidae (genus level), and the Nematoda (phylum level). Also in this case, two variables were selected as descriptors. The number of species present in each sample (S_I), as a richness measure of the invertebrate community, and the first component of a PCA (PC_{1I}) derived from the invertebrate density data. Complementary variables describing the invertebrate community structure, such as the percentage of chironomids, or the percentage of worms were also estimated.

2.3. Data analysis

The potential relationships between biofilm and invertebrate metrics with land uses occupation, physical and chemical parameters, and organic micropollutants grouped in families of products, were first explored using Pearson correlation. Variables were previously inspected for normality, and when necessary transformed using decimal logarithms. Further, some bivariate relationships were carefully described amongst the ones significant in the correlation analyses. These expressions were used to define the patterns of variation of the biological variables against the pollutant and environmental variables in the whole river data set. Sigmaplot 11 was used to define the best fitted regression curves of the biological variables with respect to the significant non-biological.

Redundancy analysis (RDA) was used to detect the ordination of the biological variables with respect to all others. RDA is a direct ordination analysis that selects a set of variables (predictors) that best explains the variance of the biological communities. RDA was performed with CANOCO for Windows (version 4.5, Microcomputer Power, Ithaca, NY, USA). The maximum gradient length for biological data was previously determined using detrended correspondence analysis (DCA). The maximum amount of variation was 1.4 standard deviation units, indicating that linear methods would be appropriate (ter Braak and Smilauer, 2002). To avoid correlation and co-linearity, variables were selected based on the inspection of non-significant correlation and variance inflator factor (VIF<20) (ter Braak & Verdonschot, 1995). This resulted in the selection of water flow (Q), variation coefficient of water flow (Q(cv)), water temperature, DOC, dissolved inorganic nitrogen (DIN), water conductivity, total phosphorus (TP), and the proportion of the different land uses, for physical and chemical variables. Only the families of organic microcontaminants showing significant correlations with the biological variables were selected to participate in the general RDA. Once defined the general RDA, a set of partial RDAs was also performed to understand the fraction of the variance that could be attributed to each of the three groups of non-biological variables (land use patterns, environmental variables, micropollutant variables).

3. Results

3.1. Land uses and hydrological characteristics

Forest was the most prevalent land use (around 60% of the surface area) in the four basins, while agriculture was the second in relevance in the Ebro, Guadalquivir and Júcar. The highest proportion of urban and industrial land cover was in the Llobregat basin, and the lowest was in the Júcar (Table 1). The Llobregat was also the most densely populated, particularly in the most downstream site (Table S1). The surface area of artificial land was associated to high water flow, temperature and water conductivity (Pearson correlations, Table 2) and higher DIN, and DOC. These areas were also significantly correlated to the higher concentration of several alkylphenols, flame retardants and anticorrosives, PCPs, and pharmaceutical products (Table 2). Agricultural land uses were associated to higher water flow and temperature, and DOC, as well as to several microcontaminants (Table 2).

The two study periods were distinctly humid (2010) and dry (2011) in all four basins, except in the Júcar (Table 1). The Llobregat and Guadalquivir rivers had much higher flows in summer 2010, and particularly the Llobregat showed coefficients of variation of water flow (Q (cv)) up to 100% (Table 1), characteristic of flood events occurring in that period. The Q (cv) was the highest in the Llobregat, and minimum in the Ebro (Table 1). Water flow was significantly associated (Pearson correlation) with increasing DIN and DOC ($r = 0.68$, and $r = 0.60$, respectively), as well as with several families of contaminants (e.g. $r = 0.49$ with flame retardants; $r = 0.46$ with diuretics, Table 2). Higher Q (cv) was mainly associated with antibiotics of human use ($r = 0.40$) and with some families of pharmaceuticals (e.g. $r = 0.53$ with psychiatric drugs).

3.2. Chemical characteristics

Water conductivity and nutrient concentrations generally increased in a downstream direction in the four basins. Maximum DIN and TP concentrations occurred in the downstream sites of the Llobregat (11.9 mg L^{-1} and 2.7 mg L^{-1}) and Guadalquivir (10.2 mg L^{-1} and 0.6 mg L^{-1}). Maximum DOC concentrations occurred also in the Llobregat and Guadalquivir (10.2 mg L^{-1} and 9 mg L^{-1} respectively).

A total of 157 organic compounds were detected in the water samples collected in 2010 and 2011. The number of detected contaminants in each site ranged between 73 and 120, for a total of 187 (2010) and 205 (2011) analyzed. Forty-two of the detected contaminants were pesticides, including thirteen herbicides, and the rest being insecticides, fungicides, nematicides and bird repellents.

Table 1
Hydrological characteristics of each of the sites included in the study. Features are provided separately for summer 2010 (wet period) and summer 2011 (dry period). The values of mean water flow, minimum flow and maximum flow (m^3 s^{-1}), and coefficient of variation (CV, percent), are provided for the period between early summer and early fall. The channel width (m) of the sites is also indicated.

	Mean flow	Min flow	Max flow	CV	Channel width
LLO3-2010	10.95	3.17	262.18	109.29	11
LLO4-2010	10.93	7.36	87.50	43.28	12
LLO5-2010	11.40	1.48	130.67	111.62	30
LLO6-2010	13.70	3.97	328.01	111.61	27
LLO7-2010	13.11	3.80	314.08	111.73	25
EBR1-2010	1.82	0.73	4.31	11.77	3
EBR2-2010	35.22	12.95	144.70	16.18	47
EBR3-2010	35.22	12.95	144.70	16.18	65
EBR4-2010	58.09	25.50	291.87	11.32	70
EBR5-2010	101.51	44.56	510.06	11.31	72
JUC1-2010	1.36	0.65	2.85	19.86	4
JUC2-2010	4.31	2.08	9.05	17.70	11
JUC4-2010	3.53	2.91	4.40	3.03	10
JUC6-2010	9.09	0.57	15.70	18.73	11
JUC7-2010	13.64	0.85	23.55	19.05	13
GUA1-2010	20.03	4.51	51.16	38.16	5
GUA2-2010	35.71	8.05	91.22	38.16	34
GUA3-2010	42.55	9.59	108.69	18.70	33
GUA4-2010	44.85	10.10	114.56	18.70	34
LLO3-2011	4.46	2.83	6.79	16.66	11
LLO4-2011	5.77	3.12	7.62	13.40	12
LLO5-2011	5.01	3.17	7.62	18.47	30
LLO6-2011	5.03	3.19	7.65	18.47	27
LLO7-2011	5.28	3.35	8.04	20.51	25
EBR1-2011	0.71	0.60	1.17	7.01	3
EBR2-2011	1.97	1.81	2.65	1.66	47
EBR3-2011	1.97	1.81	2.65	1.66	65
EBR4-2011	31.46	28.80	42.30	1.21	70
EBR5-2011	49.65	45.46	66.77	1.21	72
JUC1-2011	2.24	1.79	2.72	11.23	4
JUC2-2011	2.35	1.87	2.85	10.82	11
JUC4-2011	2.57	2.19	3.11	4.00	10
JUC6-2011	2.46	0.60	5.41	44.65	11
JUC7-2011	4.22	1.03	9.28	50.73	13
GUA1-2011	14.65	3.37	37.20	32.67	5
GUA2-2011	26.11	6.00	66.34	32.67	34
GUA3-2011	31.12	7.15	79.04	32.67	33
GUA4-2011	32.80	7.54	83.31	32.67	34

Fourteen compounds were alkylphenols, flame retardants, bisphenol-A, and anticorrosion compounds. Twenty compounds were personal care products (PCPs), including bactericides, preservatives, and UV-filters. Antibiotics included twelve compounds from eight different families, and the pharmaceutical products (PhCs) gathered sixty-nine compounds including analgesic and anti-inflammatory drugs, anticoagulants, antihypertensive drugs, β-blockers, diuretics, histamine analogue compounds, lipid regulators, psychiatric drugs and pharmaceuticals for veterinary use. The most abundant organic compounds in all the studied basins were alkylphenols, flame retardants, bisphenol-A,, anticorrosion compounds, PCPs, and PhCs. Insecticides and herbicides were found in lower concentrations, but occasionally reached presence (particularly of carbamates and fungicides) of up to 400 ng L^{-1}. The total PhCs concentrations ranged from 40 to 3000 ng L^{-1}, and those of alkylphenols, flame retardants, bisphenol-A, and anticorrosion compounds from 50 to 2300 ng L^{-1}. Within PhCs, analgesics and anti-inflammatories reached 5–510 ng L^{-1}, and diuretics reached concentrations of 100–420 ng L^{-1}. Anti-hypertensive drugs reached maximum concentrations within the range of 180–650 ng L^{-1}. Finally, high levels of alkylphenols and their ethoxylated derivatives, bisphenol A, trialkyl phosphates or benzotriazoles were detected in all basins.

The organic micropollutants differed among basins and periods. The Llobregat River showed the highest concentrations of organic micropollutants, which ranged from 1000 to 12,000 ng L^{-1}. In the Ebro the range of concentrations was of 500–1800 ng L^{-1}, 600–1400 ng L^{-1} in the Júcar and 240–2800 in the Guadalquivir. The maximum concentration of compounds was that of alkylphenols, flame retardants and anticorrosives in site LLO7 in 2011 (11,000 ng L^{-1}). PhCs were the most common organic compounds in the two periods in the Llobregat, while herbicides, pesticides and alkylphenols, flame retardants and anticorrosives presented slightly higher concentrations in the drier period (2011). The Llobregat had high levels of almost all PhCs' families. The major organic micropollutants in the Ebro were also alkylphenols, flame retardants and anticorrosives (particularly in site E3), though in lower concentrations than in the Llobregat. Herbicides in the Ebro were in similar concentrations in the two periods, but insecticides, PCPs, antibiotics and PhCs presented higher concentrations in the wet period. The Júcar had the highest pesticide concentrations of the four basins; fungicides, herbicides and insecticides were detected at high concentrations in this river, especially during the wet period. Antibiotic occurrence was similar in the two periods, but fluoroquinolones (site J5; 109.5 ng L^{-1}) and nitroimidazoles (site J3; 66.3 ng L^{-1}) were remarkably high in the dry period. The most important organic micropollutants in the Guadalquivir River were the alkylphenols, flame retardants and anticorrosives. These compounds did not show any significant difference between the studied periods, but a particular increase in site G2 in the dry period.

3.3. Biological characteristics: biofilms

Preliminary selection of the available metrics was performed by means of correlation analyses with the different stressors. This discarded the number of diatom species as a suitable descriptor, and pointed the PC_{1D}, chlorophyll-*a*, and alkaline phosphatase activity as good biological descriptors of the environmental gradient. The PC_{1D} of the diatom taxa arranged the taxa occurring in less to highly impaired sites along the first axis of the PCA (38% of the total explained variance). Some taxa (*Achnanthidium pyrenaicum*, *Achnanthidium minutissimum*, *Encyonopsis microcephala*) were characteristic of the Júcar, Ebro and Guadalquivir upper reaches, and were opposed in the PCA to *Navicula* and *Nitzschia* species (*Eolimna subminuscula*, *Navicula recens*, *Nitzschia insconspicua*, *Nitzschia palea*, *Nitzschia frustulum*) characteristic of the downstream sites of the Ebro, Llobregat and Guadalquivir. This arrangement of diatom taxa reflected the general conditions of the sites.

The microcontaminants related to the PC_{1D} were the neocotinoids, lipid regulators, β-blockers, psychiatric drugs, alkylphenols, flame retardants, anticorrosives, and bisphenol A (BPA). In all the cases, the quality pattern defined by the positive values of the PC_{1D} decreased accordingly to the increasing concentration of these products (Table 3). The PC_{1D} followed an exponential decay curve with the DIN concentration ($r^2 = 0.518$), and total phosphorus ($r^2 = 0.607$) (Fig. 2). The relationship between the diatom community and the surface area of artificial land use followed a decreasing linear expression ($r^2 = 0.66$, $p < 0.0001$). PC_{1D} was correlated linearly with several organic contaminants ($r^2 = 0.272$ with diuretics; $r^2 = 0.452$ with anticorrosive products) (Fig. 3).

The alkaline phosphatase activity (APA) was also inversely related to several micropollutants (e.g. analgesic and anti-inflammatories, lipid regulators, antihypertensives, diuretics, flame retardants, and parabens) (Table 3). The expressions were linear in most cases ($r^2 = 0.378$, $p < 0.0001$ with diuretics, $r^2 = 0.452$, $p < 0.0001$ with) (Fig. 4). There was a coincident negative relationship of several descriptors of the biofilm with microcontaminants occurrence; APA and PC_{1D} showed analogous

Table 2
Correlations (Pearson) between land uses, environmental variables and microcontaminants (n = 38). The significant results with p values < 0.05 are indicated in italics, and those with p values < 0.01 are highlighted in bold. Those variables without any significant correlation are not shown.

	Artificial	Agricultural	Water flow	Q(cv)	Cond	Temp.	DIN	TP	DOC
Water flow	**0.61**	**0.48**			**0.46**	*0.4*	**0.56**		**0.59**
Q(cv)					*0.40*		**0.47**		*0.41*
Conductivity	**0.62**	*0.38*	**0.46**	*0.40*		**0.45**	**0.68**	**0.42**	*0.39*
Temperature	**0.77**	**0.61**	*0.40*		*0.45*		**0.48**	*0.36*	*0.38*
DIN	**0.48**		**0.56**	**0.47**	**0.68**	**0.48**		**0.55**	**0.48**
TP	*0.37*				**0.42**	*0.36*	**0.55**		*0.36*
DOC	**0.58**	**0.41**	**0.59**	**0.41**	0.39	0.38	**0.48**	0.36	
Herbicides					**0.45**	*0.34*	**0.63**	**0.62**	
Azoles					*0.34*				**−0.42**
Neocotinoids	*0.4*				*0.39*	**0.53**	*0.34*	**0.66**	
Miscellaneous pesticides					*−0.32*	**−0.43**			
Antibiotics				*0.40*		*0.33*	*0.4*	**0.45**	
Analgesic and anti-inflammatories	*0.39*					*0.35*		**0.47**	*0.37*
Anticoagulants								**0.45**	
Lipid regulators	**0.6**	*0.35*			**0.59**	**0.5**	*0.4*	**0.62**	**0.42**
Histamines						**0.43**		*0.38*	
B-blockers						*0.4*		**0.7**	*0.41*
Antihypertensive			**0.41**	**0.50**					**0.68**
Diuretic	**0.53**		**0.46**	**0.48**	*0.38*	**0.47**	**0.46**	**0.46**	**0.58**
Psychiatric drugs	*0.4*			**0.52**	*0.39*		*0.39*	**0.51**	**0.61**
Alkylphenols	**0.54**	*0.36*	**0.43**		**0.59**	*0.37*	**0.55**	**0.54**	**0.58**
Flame retardants	**0.61**	*0.39*	**0.49**	*0.40*	**0.65**	**0.46**	**0.68**	**0.61**	**0.58**
Anticorrosives	**0.64**				**0.48**	**0.67**	*0.4*	**0.52**	
Bisphenol A (BPA)	*0.39*				**0.57**		*0.38*	*0.38*	
UV filters	*0.4*				**0.5**		**0.52**	**0.59**	

correlation trends in relation to water flow, lipid regulators, diuretics, and flame retardants (Table 3; Fig. 4).

3.4. Biological characteristics: invertebrates

The richer invertebrate community composition was in the upstream sites of the four rivers. *Leuctra* sp., *Elmis* sp., *Atrichops* sp., *Rheocricotopus* spp., *Parametriocnemus* sp, and *Lumbriculus variegatus* were common in these sites. *Chironomus* sp., *Limnodrilus hoffmeisteri*, *Branchiura sowerbyi* or *Caenis luctuosa* were abundant in the downstream sites affected by the general impairment. The invertebrate community therefore had higher species richness (S_I) in the upstream sites and much lower richness in the impaired sites. Collectors (mostly collectors-gatherers) were the dominant feeding strategy in the invertebrate community, represented by worms and chironomids. A Principal component analysis carried out with the most frequent invertebrate taxa defined an ordination in the first principal component (PC_{1I}) similar as the one described above: those species sensitive to pollution (e.g. *Leuctra* sp., *Elmis* sp., *Atrichops* sp., *Parametriocnemus* sp., *Lumbriculus variegatus*) and present in upstream sites were separated from those more abundant in impaired sites downstream (*Orthocladius* spp., *Thienemannimyia* sp., *Limnodrilus hoffmeisteri*, *Branchiura sowerbyi*, *Caenis luctuosa, Corixa* sp., *Micronecta* sp.).

Preliminary correlation analyses using the available biological descriptors with the non-biological variables led to non-significant results for the PC_{1I} of the invertebrates. This variable was therefore discarded as a suitable descriptor of the invertebrate response to impairment. While the PCA is useful to summarize species distribution patterns, it considers both their occurrence as well as their abundance with respect to pollution. This potentially more complex approach did not correlate with any of the stressor variables. The percentage of chironomids and the percentage of worms were not either related to any of the variables associated to the environmental gradient, and therefore were not considered for further analyses. The variable retained as a descriptor of the invertebrate response was therefore S_I, the diversity of the invertebrate community (Table 3). S_I decreased with increasing DIN concentration ($r = -0.51$), DOC concentration ($r = -0.47$) and overall water conductivity ($r = 0.47$). S_I showed a linear regression with most of the environmental descriptors of the sites. This was the case for DIN ($r^2 = 0.213$), and weakly for DOC ($r^2 = 0.154$) and total phosphorus ($r^2 = 0.105$; Fig. 2). The microcontaminants more related to the diversity of invertebrates (S_I) were the lipid regulators, antihypertensives, diuretics, alkylphenols, flame retardants, anticorrosives,

Table 3
Correlations (Pearson) between land uses, environmental variables and organic microcontaminants with respect to the selected biological variables (the first component of the diatom community analysis PC_{1D}, the alkaline phosphatase activity (APA), and the diversity of the macroinvertebrate community S_I). Significant results with p values < 0.05 are indicated in italics, and those with p < 0.01 are highlighted in bold. Those variables without any significant correlation are not shown.

	PC_{1D}	APA	S_I
Artificial surface area	**−0.81**	*−0.35*	**−0.69**
Agricultural surface area	**−0.44**		**−0.59**
Conductivity	**−0.71**		**−0.47**
Temperature	**−0.71**		**−0.69**
DIN	**−0.63**	*−0.34*	**−0.51**
TP	**−0.44**		
DOC	**−0.59**	*−0.39*	**−0.47**
Water flow	**−0.65**	**−0.51**	**−0.58**
Herbicides	*−0.35*		
Organophosphorates		*0.39*	
Neocotinoids	**−0.55**		
Antibiotics	−0.31	*−0.34*	
Analgesic and anti-inflammatories	−0.4	**−0.53**	
Lipid regulators	**−0.62**	**−0.47**	
B-blockers	**−0.42**		
Antihypertensives	−0.36	**−0.49**	*−0.41*
Diuretics	**−0.62**	**−0.6**	*−0.44*
Psychiatric drugs	**−0.43**	*−0.4*	
Alkylphenols	**−0.58**	*−0.36*	*−0.46*
Flame retardants	**−0.62**	**−0.5**	*−0.42*
Anticorrosives	**−0.67**		**−0.51**
Bisphenol-A (BPA)	**−0.52**		*−0.44*
UV-filters	**−0.42**		
Parabens		**−0.41**	

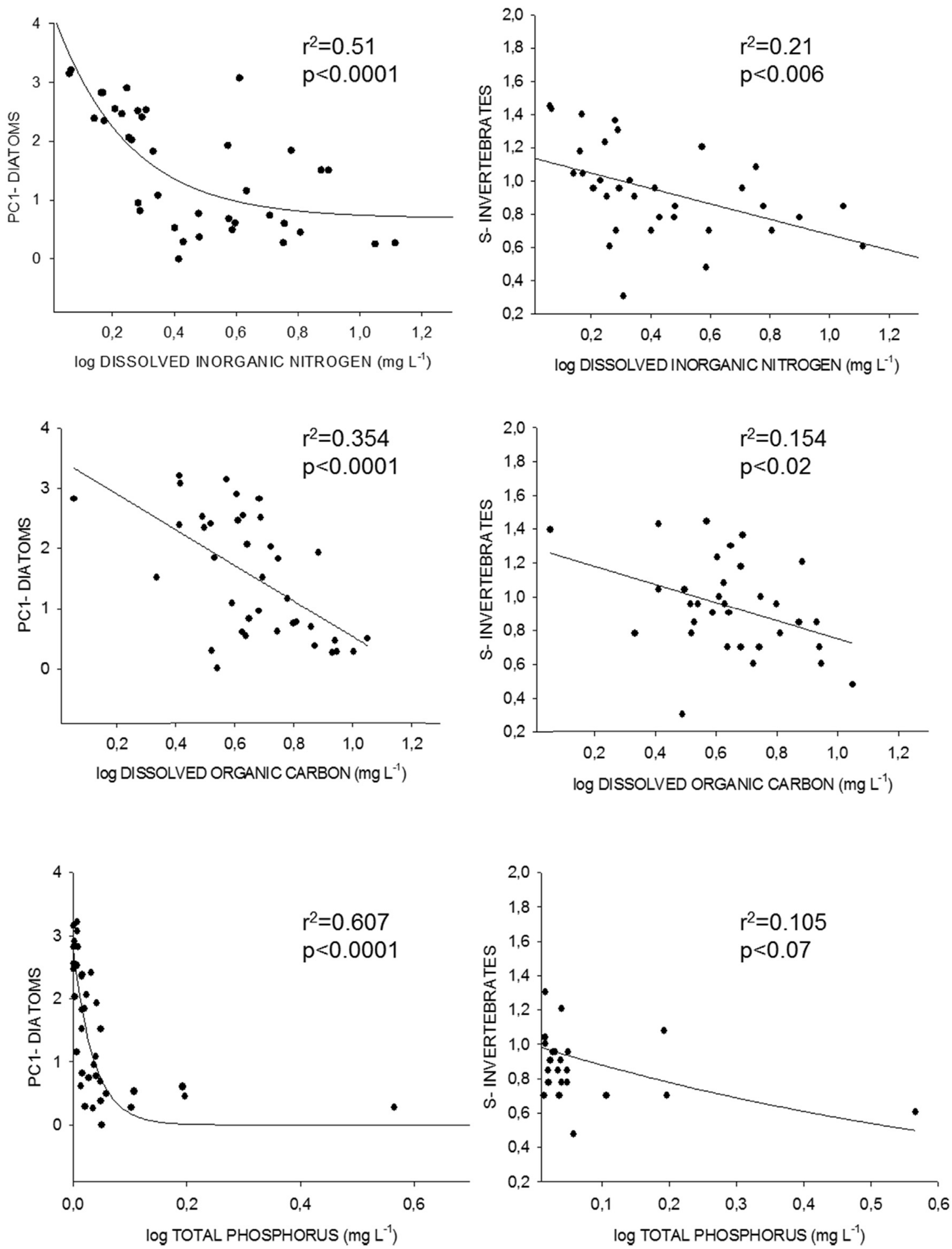

Fig. 2. Relationships between the PC_{1d} (left) of the diatom communities in the biofilm and the number of species of invertebrates (S_I) (right) with several environmental descriptors (dissolved inorganic nitrogen, dissolved organic carbon, total phosphorus). The best fit, the r^2 and the probability of each expression are also indicated.

and bisphenol A (BPA) (Table 3). The number of species decreased linearly according to the increasing concentration of these products (Fig. 3).

3.5. Joint data analysis

An RDA was performed using the diatom community composition (PC_{1D}) and the invertebrate community diversity (S_I) as selected metrics of the biological communities' structure (Fig. 5). These two metrics were used as fixed variables against the physical and chemical variables selected by the analysis. The first axis of the RDA accounted for the 76.4% of the variance and indicated an analogous general response of PC_{1D} and S_I with respect to the environmental gradient (Fig. 2). The two biological descriptors

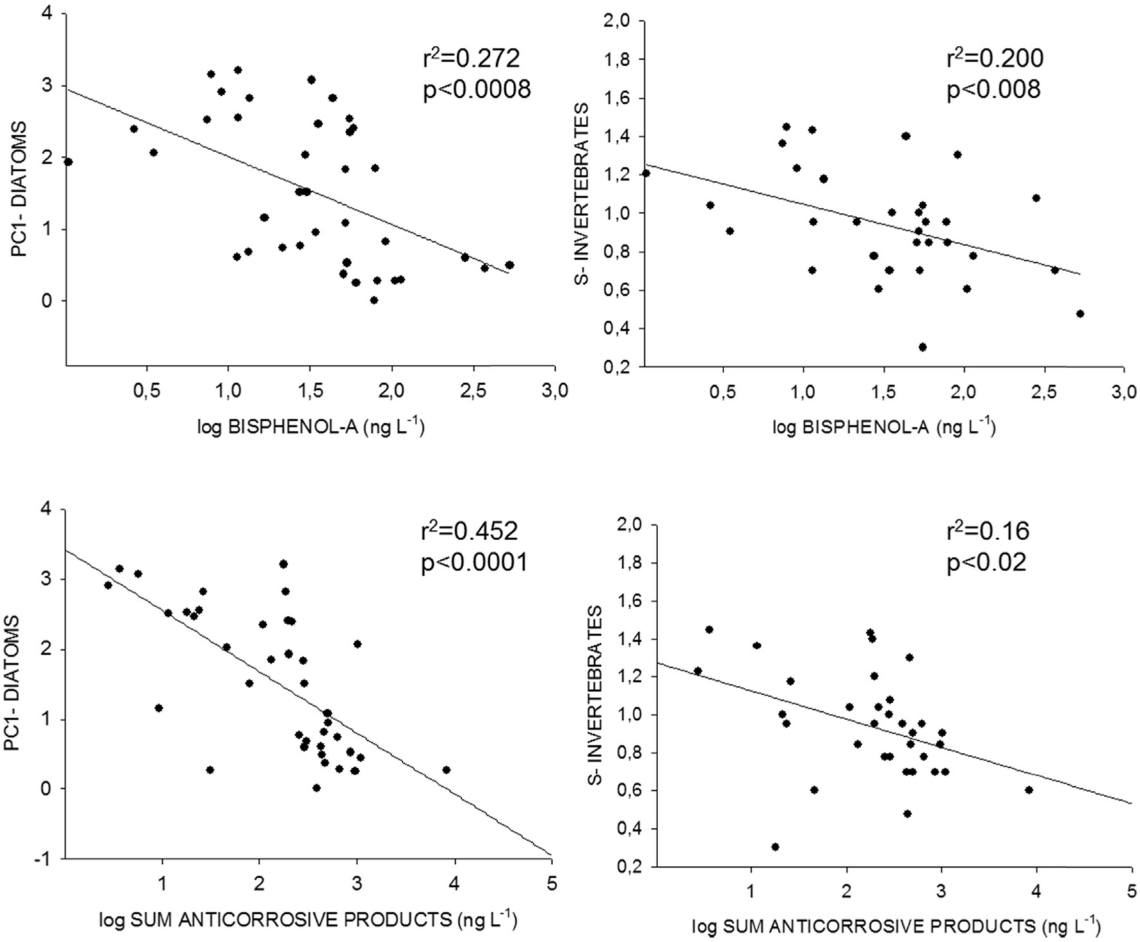

Fig. 3. Relationships between the PC_{1d} (left) of the diatom communities in the biofilm and the number of species of invertebrates (S_I) (right) with several organic micro-contaminants (Bisphenol-A and the sum of anticorrosive products). The best fit, the r^2 and the probability of each expression are also indicated.

were opposed to DIN, increasing water flow, and higher surface area of agricultural lands. PC_{1D} and S_I were also opposed to BPA, lipid regulators, anticorrosives, and artificial (urban and industrial) land use. The position of the diatoms vector PC_{1D} was the most opposed to all the physical and chemical variables describing multiple stress, particularly those in the downstream sites of the Llobregat, Guadalquivir and Ebro. The position of S_I was less apparently opposed to this ensemble of variables, and closer to the downstream sites of the Guadalquivir and Júcar (Fig. 5). The second axis of the RDA (5.1% of the variance) separates the upstream sites of the Guadalquivir, Júcar and Ebro from the rest.

After this general RDA a subsequent partition of the variance analysis was performed to discriminate the respective relevance of land-uses, physico-chemical, and micropollutant variables with

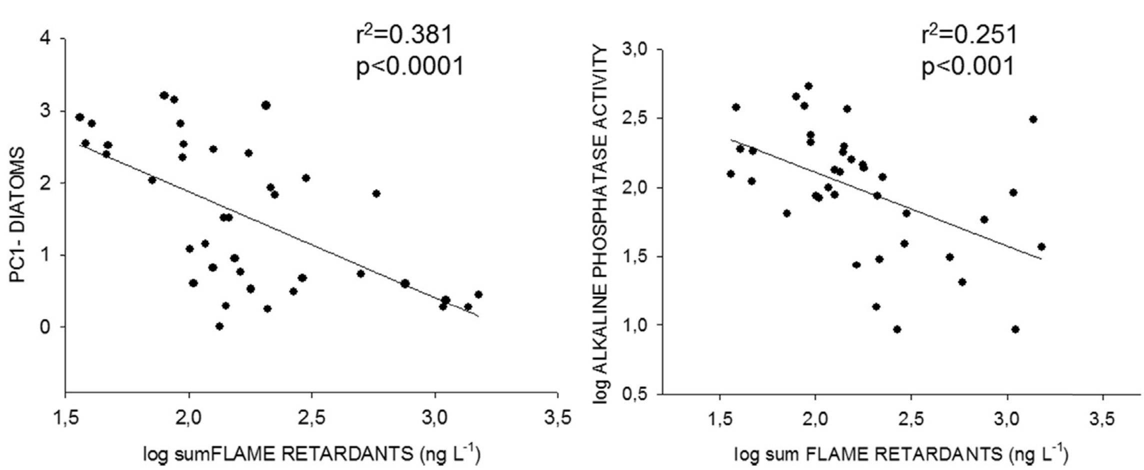

Fig. 4. Relationships between the PC_{1d} (left) and the alkaline phosphate activity (right) with flame retardants. The r^2 and the probability of each expression are also indicated.

respect to the biological variables. A set of partial RDAs were performed for all the combinations of stressors in order to account for the different interactions and shared variances. The TP, DOC, DIN, water conductivity, water temperature, and water flow were selected for the physic-chemical variables, artificial and agricultural land areas were selected for land uses, and bisphenol-A, anticorrosives and lipid regulators were selected for the group of organic micropollutants. The total explained variance was the 86.3%, where a 2.2% was directly attributed to the organic micropollutants, 5.7% to land uses, and 10.6% to the environmental variables. The shared variance between organic micropollutants and land uses was of 4.1%, but the one shared between land uses and physico-chemical variables was the 21.2%. The total shared variance of the three groups of variables was 41.3% (Fig. 6).

4. Discussion

The analysis of the data revealed that the biofilm and invertebrate community had similar and complementary responses to the stressors occurrence and relevance, with a progressive decrease in biodiversity and associated simplification of the biological structure. Nutrients and DOC in excess, higher abundance of artificial land uses, and higher concentrations of organic microcontaminants accounted, in this order, for the distribution of the two biological communities. However, most of the response of the biological metrics could not be attributed solely to one or the others, but to the joint expression of the different stressors in the sites.

The multivariate analysis (RDA) used in this study attributed a common pattern to the distribution of the algal (biofilm) and invertebrate communities, showing that they were associated to the progressive impairment of the sites. Increasing areas of agricultural, and industrial or urban lands were associated to higher inorganic nutrient concentrations, increasing dissolved organic matter, and increasing concentrations of organic micro-contaminants. The general response of the two biological communities to the progressive river impairment was towards a decrease in community diversity and to the higher occurrence of species tolerant to pollution. Even though species replacement naturally occurs along a downstream river gradient, as a response to changes in the river environment (e.g. temperature, habitat, food resources; Margalef, 1983), the ones occurring in our rivers were related to their respective tolerance to pollution. Both for the algae and invertebrates, the decline in diversity was mainly related to the decline of species non-tolerant to organic pollution, that however occur in analogous but non-polluted systems (Bennett et al., 2011; Almeida et al., 2014). Biofilms and invertebrates represent two major components of the river trophic webs (Allan and Castillo, 2007): biofilms include primary producers (algae) and heterotrophs (bacteria, fungi) in a highly cooperative consortium (Lock et al., 1984); invertebrate communities include all consumer feeding strategies, from herbivores and detritivores to predators (Anderson and Sedell, 1979). Therefore, biofilms and invertebrates are inclusive of most of the biological elements involved in the transference of energy and matter in the river. Showing a common response can be taken as an indication of the analogous effect caused by the stressors, and as an evidence of the overall effect on river biodiversity.

The physical and chemical variables selected by the multivariate analysis were the ones most relevant for the algal and invertebrate communities. The variables could be considered as stress descriptors acting in the river site. Those variables selected by the analysis were analogous to those affecting the biotic community structure in impaired rivers elsewhere. Artificial and agricultural land uses have been associated to the massive arrival of DIN to the river (Burkart and James, 1999; Nikolaidis et al., 1998; Poor and McDonnell, 2007), as well as to the continuous inputs of pharmaceutical products and other contaminants (Burkart and Kolpin, 1993; Allan, 2004). de Zwart et al. (2009) observed significant taxa loss as a result of the highly polluted conditions in the Scheldt

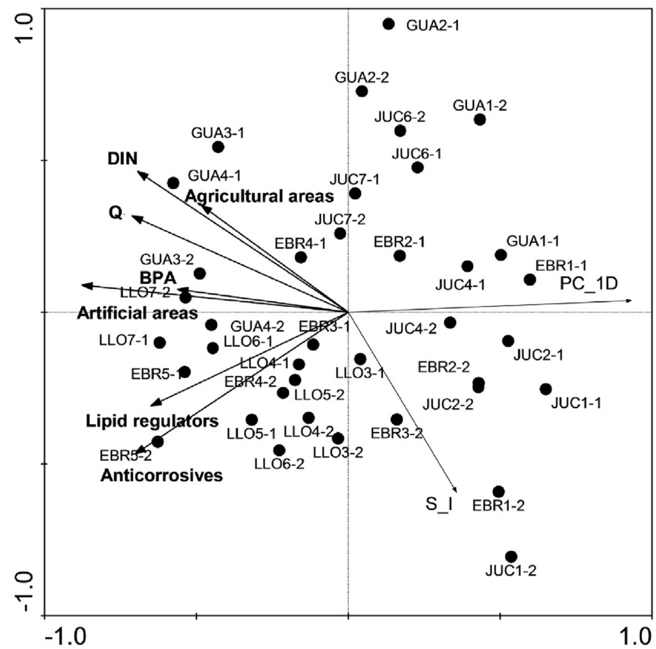

Fig. 5. RDA performed using the diatom community composition (PC_1D) and the invertebrate community diversity (S_I). The variables participating in the analysis were DIN, water flow, surface areas of agricultural and artificial lands, BPA, lipid regulators, and anticorrosives. The sites of the Llobregat (LLO), Guadalquivir (GUA), Júcar (JUC) and Ebro (EBR) of the 2010 sampling were indicated as −1, and those of the 2011 sampling as −2.

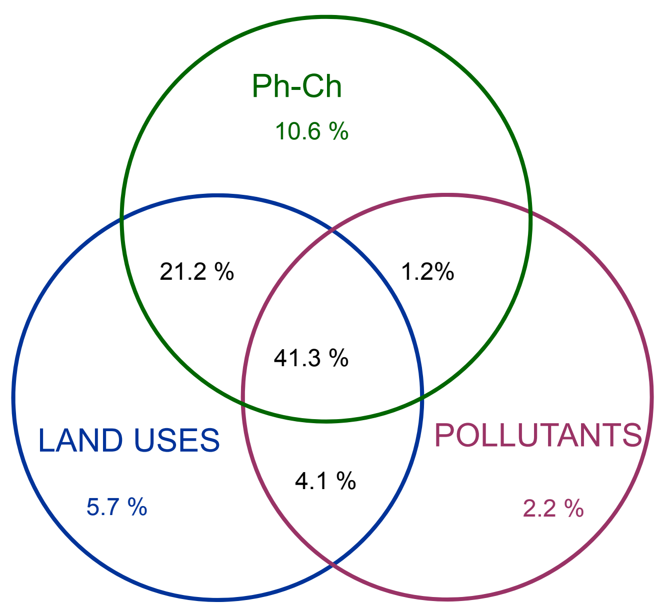

Fig. 6. Shared variance resulting from the partition of the variance analysis between physico-chemical variables (Ph-Ch), land uses, and organic micropollutants. This analysis was performed by separating land-uses, environmental, and micropollutant variables, and identifying its share with respect to the biological variables included in the study (invertebrates and diatoms).

River. Effects on species richness of benthic macroinvertebrates have been associated to sediment-bound contaminants (trace metals, polycyclic aromatic hydrocarbons, polychlorinated biphenyls) in the rivers Rhine and Meuse (De Lange et al., 2004). At larger spatial scales, the diversity and composition of algae, macrophytes, invertebrates and fish in European streams, has been associated to increasing nutrient concentrations (Johnson and Hering, 2009). Pesticide occurrence has been associated to reduced invertebrate richness in sites when concentrations are close to the legal threshold levels (Stehle and Schulz, 2015), as well as to high local losses in the invertebrate species pools (Beketov et al., 2013). All these field-derived evidences show that several sorts of variables affect the biota in impaired rivers, often coinciding in space and time, and able to produce similar consequences to biodiversity. These consequences follow a rather general mechanism: the most sensitive species are affected, even becoming locally extinct, and tolerant others are favored (Blanck et al. (1998)) up to a certain threshold. This was analogously expressed by the algae and invertebrate communities in our study set: a decrease in diversity, and biological communities made up of species tolerant to the new conditions.

The effect of stressors in our study set was not only evidenced by the biodiversity decrease of the algal and macroinvertebrate communities. The alkaline phosphatase activity (APA), an expression of the transformation of organic phosphorus into inorganic by bacteria, cyanobacteria and some algae (Chróst and Overbeck, 1987), decreased in the sites with higher concentrations of DIN and DOC and higher concentrations of organic contaminants such as analgesics and diuretics. The APA decrease suggests that the biofilm ability to transform organic phosphorus into inorganic (and available) phosphorus could be seriously limited in those polluted areas. Regarding the invertebrate community, De Castro-Català et al. (2015) observed significant correlation between the activity of the antioxidative enzyme catalase in the invertebrate *Hydropsyche exocellata* and the presence of EDCs and PhCs in the sites. Such a stress response on the invertebrate community has been observed also under different sources of pollution such as heavy metals (Barata et al., 2005).

The correlation analysis and the RDA revealed that effects of environmental stressors such as nutrients in excess and DOC on the distribution of the biological communities were higher than that of organic micropollutants. The partition of the variance showed a low relevance of the measured organic micropollutants on the distribution of diatom and invertebrate communities (2%), while the one corresponding to the environmental factors (nutrients, DOC, water flow) was higher (ca. 10%). Even though the multivariate analysis results need to be used cautiously, the variance of the different stressors express the relevance of factors such as irregular flow patterns, high water conductivity, and high DIN and DOC concentrations. Such a result should not be surprising according to the higher potential impact associated to the impairment of river habitat, hydrological patterns, or inorganic nutrients (Elosegi and Sabater, 2013), than the one potentially produced by organic micropollutants.

Our results do not preclude the potential of organic microcontaminants to produce particular effects on the biota. A separate analysis of the associated ecotoxicological risk of contaminants in the four studied basins, based on the toxic units (TU) approach, was performed by Kuzmanovic et al. (2015) using the same sampling scheme than ours. TUs for individual contaminants were calculated using algae (*Scenedesmus*) and invertebrates (*Daphnia*), and then aggregated under the assumption of concentration addition (CA) to derive the site specific risk. Their risk assessment analysis indicated that organic chemicals were able to pose risk of acute effects at 42% of the sampling sites, and chronic effects to all of the studied sites, particularly to invertebrates. The higher potential toxicity (TU values of −0.28 to −1.27) was estimated in sites showing the highest concentrations of pesticides. The correlation results of our invertebrate and algal metrics identified some microcontaminants also identified by the risk assessment. Gemfibrozil was identified to show high risk for the biota (Kuzmanovic et al., 2015), and we also found it to be significantly associated to poorly diverse biofilms communities and low alkaline phosphatase activity. Gemfibrozil is the lipid regulator most abundant in our series of polluted sites, and has been described to induce transcriptional responses of several bacterial genes involved in lipid metabolism (Yergeau et al., 2010), an early indication of potential more severe effects on biofilms. Flame retardants were also identified by the two approaches. These contaminants have been pointed out as disruptors of invertebrate development (Wallstrom et al. 2005), as well as able to produce adverse effects on biofilm algae in locations close to industrial and urban sewage discharges (Cristale et al., 2013). Even though a tight coincidence of the two approaches should not be expected since they provide different perspectives (an *a priori* estimation of chemical effects not necessarily coinciding with real ecosystem effects, and an estimation of the relevance of factors performing as stressors on the biota, respectively), the coincidence highlights the real relevance of these contaminants in the ecosystem.

Overall, our analysis indicates that the organic micropollutants mainly affect the distribution of organisms already affected by other stressors (Allan et al., 2013; Coors and DeMeester, 2008), or the other way round. The partial RDAs show the very high fraction of the variance (nearly half of the total explained) shared between the organic micropollutants and the remaining environmental stressors, pointing to their common relevance for the distribution of the biological communities. Environmental stressors may reinforce the effect of organic micropollutants, or vice-versa (Segner et al., 2014). Stressors occurring at multiple spatial and temporal scales define a so-called “stressor space” were the net receivers are the biological communities, and where synergies could produce much higher effects than the ones attributed solely to organic microcontaminants or to inorganic nutrients. Whatever the causes, it is obvious that the multiple and simultaneous occurrence of multiple stressors challenges the carrying capacity of ecosystems (Posthuma et al., 2014) by affecting their biodiversity and basic functions. Understanding the real risks affecting the biological communities requires quantifying the effects of multiple stressors in impaired systems.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2016.01.037.

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